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# Programming effects of an early-life diet containing large phospholipid-coated lipid globules are transient under continuous exposure to a high-fat diet

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**Short title:** Long-term effects of early life diet

**Abbreviations:** IMF: infant milk formula, eIMF: experimental IMF, cIMF: control IMF, PN: postnatal, HFD: high fat diet, GTT/ITT/PTT: Glucose/ Insulin/ Pyruvate Tolerance Test, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, CM: chylomicrons

**Keywords**: metabolic programming, infant nutrition, dietary lipids, lipid structure, milkfat globule membrane, obesity prevention, animal model

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## Abstract

Breastfeeding is associated with a lower risk of developing obesity during childhood and adulthood compared to feeding infant milk formula (IMF). Previous studies have shown that an experimental IMF (eIMF; comprising Nuturis®), programmed mouse pups for a lower body weight and fat mass gain in adulthood when challenged with a high-fat diet (HFD), compared to a control IMF (cIMF). Nuturis has a lipid composition and structure more similar to breastmilk. Here, the long-term effects were tested of a similar eIMF, but with an adapted lipid composition, and a cIMF, on body weight, glucose homeostasis, liver and adipose tissue. Nutrient composition was similar for the eIMF and cIMF; the lipid fractions comprised ~50% milkfat. C57BL/6JOlaHsd mice were fed cIMF or eIMF from postnatal (PN) day 16-42 followed by a HFD until PN168. Feeding eIMF versus cIMF in early life resulted in a lower body weight (-9%) and body fat deposition (-14%) in adulthood (PN105). The effect appeared transient, as from PN126 onward, after 12 weeks HFD, eIMF-fed mice caught up on controls and body and fat weights became comparable between groups. Glucose and energy metabolism were similar between groups. At dissection (PN168), eIMF-fed mice showed larger (+27%) epididymal fat depots and a lower (-26%) liver weight without clear morphological aberrations. Our data suggest the size and coating but not the lipid composition of IMF fat globules underlies the programming effect observed. Prolonged exposure to a HFD challenge partly overrules the programming effect of early diet.


## Introduction

Breastfeeding is epidemiologically associated with a lower incidence in childhood and adulthood obesity, compared to infant milk formula (IMF)-feeding (1). Breastfeeding is also associated with lower blood pressure and lower plasma cholesterol levels in adulthood (2, 3). The nutritional composition of human milk (HM) is mimicked in IMF. However, mimicking fat emulsification is not yet possible. Fat in HM is dispersed in particles (5 µm diameter) enveloped by a trilayered milk fat globule membrane, composed mainly of phospholipids and cholesterol (4). In contrast, standard IMF fat globules are much smaller (0.1 µm diameter) and coated with surface-adhering proteins (4, 5). Most likely the physicochemical structure (size and coating) of lipid globules in HM serves a biological purpose (4-8). An experimental IMF (eIMF; Nuturis®) was developed comprising large (mode diameter 3-5 µm) phospholipid-coated lipid globules similar in size to those in HM (4, 6). Feeding mice an eIMF-based diet in early life, compared to standard control IMF (cIMF), resulted in a lower body weight and lower fat mass accumulation when fed a high-fat diet (HFD) challenge diet into adulthood (6, 9, 10). The underlying mechanism of this effect on fat deposition of eIMF exposure has not yet been elucidated, but is hypothesized to relate to fat globule size and structure.

To confirm and extend previous observations on eIMF programming effects in mouse pups, we used a similar paradigm, and determined the possible long-term effects of early-life eIMF exposure on body weight accrual, glucose homeostasis, and liver and adipose tissue attributes. We tested the hypothesis that the size and coating of lipid globules, and not the composition, underlies the initial programming effect observed. We therefore compared the cIMF and the eIMF with similar lipid compositions.

## Materials and methods

**Animals and Study design** Experimental procedures were approved by an external independent animal experiment committee (Central Animal Experiments Committee, The Netherlands), and complied with the principles of good laboratory animal care following the EU-directive for the protection of animals used for scientific purposes. This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Ethics Committee for Animal Experimentation of the University of Groningen (NVWA 10500) in full compliance to the European Directive 2010/63/EU for the use of animals for scientific purposes. All animals were kept in the same temperature-controlled room (21±1°C, 55±10% humidity, lights on 8AM-8PM) in type 1L (360 $cm^2$) polysulfone cages bearing stainless-steel wire covers (UNO BV, the Netherlands), with wood shaving bedding, Enviro-dri® (TecniLab, The Netherlands) and cardboard rolls. All mice were handled by the same researcher. Virgin C57BL/6JOlaHsd breeders (11M, 22F) 12 weeks of age, Envigo, The Netherlands) were mated (6) in 2F+1M couples. Males were removed from couples after 2 d. Pregnancy was confirmed by a >2 g increase in body weight after 1 week, and occurred at ~66% efficiency. Delivery day was recorded as postnatal day (PN) 0. Pups were randomized between dams, and litters were culled to 4M+2F on PN2, weaned at PN21, and diets provided as freshly prepared dough balls (40% water) from PN16 to PN42 (6, 9). Randomization was not performed as the programming diets were visually distinct. Breeders and female offspring were terminated ($CO_2$) at weaning, in compliance with the AVMA Guidelines for the Euthanasia of Animals. From PN42 onward, male offspring was pair-housed with siblings and fed a high-fat diet (HFD, 45%en fat, D12451 Research Diets Inc. USA) and tap water *ad libitum* until dissection on PN168. Females were not used, as they are protected against HFD-induced metabolic changes (11). Glucose tolerance and calorimetry was assessed at PN133 and PN154, respectively (Fig 1). **Programming diets** Two IMF powders (Nutricia Cuijk B.V., Cuijk, the Netherlands) were tested. The IMF powders had a similar macro- and micronutrient content (Table 1); both lipid moieties comprised about 50% vegetable oil and 50% milkfat (Table 1) and had a similar fatty acid profile (Table 2). cIMF comprised fat globules with a volume mean diameter (D[4,3]) of 0.8 µm, whereas eIMF comprised phospholipid-coated lipid globules with a D[4,3] of 7

µm, explained in more detail elsewhere (4). IMF powders (283 g/kg feed) were supplemented with protein and carbohydrate (Ssniff Spezialdiäten GmbH, Soest, Germany) to obtain AIN-93G-compliant diets, with a fat moiety derived entirely from IMF (12).

**Body composition** Lean and fat mass was quantified by time-domain nuclear magnetic resonance (LF90II, Bruker Optics, Billerica, MA), not requiring fasting or anesthesia. **Glucose, insulin and pyruvate tolerance tests** Mice were fasted 6, 6 and 4 hours for the glucose, pyruvate and insulin tolerance test (GTT, PTT, ITT), respectively. GTT (i.p. 13.9 µmol glucose/g BW), ITT (i.p. 0.5 mU insulin/g BW) and PTT (i.p. 28.4 µmol pyruvate/g BW) were performed as previously described (13). C**alorimetry** Mice were single-housed in a Comprehensive Laboratory Animal Monitoring System (Phenomaster, TSE systems GmbH, Bad Homburg, Germany) at PN154 for 4 days as previously described (14). **Termination** Mice were anaesthetized (isoflurane/$O_2$) after a 4-h fasting period (during light phase) and sacrificed by heart puncture; a terminal blood sample was drawn. Liver, epididymal, inguinal, perirenal and interscapular fat was obtained and weighed. **Assays** Plasma was analyzed using the V-PLEX Proinflammatory Panel 1 (mouse) kit (K15048D), Mouse Adiponectin Kit (K152BXC), Mouse Leptin Kit (K152BYC), Mouse MCP-1 Ultra-Sensitive Kit (K152AYC), Mouse/Rat Total Active GLP-1, Insulin, Glucagon Kit (K15171C) and the Mouse/Rat Resistin Kit (K152FNC). Analyses were performed according to the manufacturer's instructions. Kits were purchased from MSD (Meso Scale Diagnostics LLC, USA). Blood glucose was measured using a OneTouch Select Plus (Lifescan Inc., USA).

**Liver fatty acyl chain profiling** Cryogenically crushed liver was homogenized in Potter-Elvehjem tubes. Lipids were trans-methylated, extracted and analyzed by gas chromatography as previously described (15).

**Analysis of gene expression** Gene expression was analyzed by quantitative real-time PCR as previously described (16). Cyclophilin and 36b4 were used as housekeeping genes for hepatic and adipose tissue gene expression respectively. Primer and TaqMan probe sequences are given in Suppl Table 1.

**Histological analysis** A liver lobe and the left epididymal fat pad were formalin-fixed and paraffin-embedded, sectioned, H&E stained. Liver slices were scored blindly for steatosis, NAS (17), ballooning (18) and findings were reviewed by a certified veterinary pathologist (AdB). Liver sections were stained for the proliferation marker Ki-67 as previously described (19). Histological scoring of Ki-67 was performed in 5 separate x40 fields by a single assessor. Binucleation and karyomegaly was assessed as described (20). Adipose tissue sections were quantified using Adiposoft (21). Adipose tissue was assessed for the presence of inflammatory foci ('crown-like structures') as described (22).

**Statistical analysis** Statistics were performed using GraphPad Prism 5 (GraphPad Software, USA) and SPSS 23 (SPSS Inc., USA). Data are plotted as Tukey box-and-whisker plots unless stated otherwise. Group sizes were calculated (23) using a relevant and most varied value, previously obtained (plasma IL-6) (6); expected difference 12 ng/L, spread 10 ng/L, alpha 0.05, beta 0.80. Analyses were carried out on all individuals whenever material was available and no outliers were excluded.

## Results

### eIMF transiently lowers body weight gain on HFD

An initially comparable rapid weight gain (PN42-56) preceded a period (PN63-119) of lower weight gain in eIMF compared to cIMF (-9% on average, $p<0.05$). From PN126 onwards, weights between groups were no longer significantly different (Fig. 2A). Fat and lean mass were similar at PN42. At PN72, 105 and 126, fat mass was substantially lower in eIMF compared to cIMF (-10%, -14%, -7%; $p<0.01$), and lean mass was slightly lower (-4%, -8%, -6%; $p<0.01$; Fig 2B) respectively. Fat percentage and lean percentage were similar at PN42. The average fat percentage (PN42-126) was not-significantly lower in eIMF (-5%; $p=0.07$), whereas lean percentage was not-significantly higher in eIMF (+3%; $p=0.08$; Fig. 2C). To assess whether later-life effects were due to differences in growth in early life, we measured body weight from weaning, which was similar between groups, and body composition at PN28 and 35; which was similar. At PN147-154, daily food intake was on average 14% lower in eIMF ($p=0.08$, Fig. 2F), correlating with the calculated slope of prior weight gain (PN42-PN147, Spearman's rank-order, $r_s = 0.6$, $p<0.01$). Energy expenditure (Fig. 2G), and locomotor activity (Fig. 2H) were similar. At PN133 the ipGTT time course (Fig 2I) and AUC (2.1±0.6 versus 1.9±0.4 M·min) was similar between groups. The ipITT (PN140) and ipPTT (PN147) AUCs (1.4±0.3 versus 1.2±0.2 and 1.6±0.5 versus 1.4±0.4 M·min, respectively) were similar.

### eIMF-programmed mice have a lower liver weight independent of body weight

Upon dissection at PN168, we noted that eIMF-fed mice had lower liver weights (Fig 3A; -25%; $p<0.05$) and a lower liver-to-body weight ratio (Fig 3B; -23%; $p<0.01$). The lower liver weight was not related to triglyceride (TG) levels, which showed moderate variability (Fig 3C). Liver protein (mg/g liver) was higher in the eIMF group (+9%; $p<0.05$), whereas total liver protein was lower in eIMF (mg/liver; -20%; $p<0.05$, Fig 3D). Gene expression markers for hepatic *de novo* lipogenesis (Fasn, Scd1, Acaca, Pparg) and fatty-acid oxidation (Pparα, Pgc1α, Cpt1) (Fig 3E) were similar. The liver fatty acyl-chain profile (Fig 3G) was similar between groups (Fig 3G).

Histological analysis (Fig 3H, Table 3) showed that steatosis tended to be higher in cIMF (70 ± 17 versus 57 ± 32 %, steatosis grade 2.5 ± 0.5 versus 2.1 ± 1.0), but this difference did not reach statistical significance. Microvesicular steatosis was more frequent than macrovesicular steatosis in both groups and typically showed a zonal distribution characterized by central microvesicular steatosis with mild to moderate mid-zonal (occasionally extending to portal) macrovesicular steatosis. Lobular inflammation and ballooning was similar between groups. NAFLD score, mostly influenced by steatosis, tended to be higher in cIMF but did not reach statistical significance. Varying degrees of biliary / oval cell hyperplasia were seen in almost all mice. The mitotic index (Ki-67) was similar between groups. Binuclear hepatocyte counts in the central and mid/portal region were similar between groups. Hepatic fatty acyl chain ratios representing lipid-related enzymatic activity (24, 25) were similar between groups.

**Body fat storage was shifted by eIMF programming without affecting adult adipokine levels**

At PN168 we analyzed adipose tissue and plasma adipokines (Fig 4). Epididymal fat mass was larger in eIMF (Fig 4A; +27%; $p<0.01$). Inguinal fat mass was similar. Interscapular brown fat mass tended to be smaller in eIMF (-10%; $p=0.06$). The median epididymal adipocyte diameter tended to be higher in eIMF (Fig 4B; +11%; $p=0.08$). The median perirenal and inguinal adipocyte diameter (Fig 4B) was comparable. Gene expression for Ppar-γ, Fas, Fabp4, Tnf-α and Cd68 was similar between groups (Fig 4C). The assessed adipokines (leptin, adiponectin, resistin, Mcp-1, Tnf-α and Il-6), glucostatic hormones (insulin and glucagon), and cytokines (Ifnγ, Cxcl-1, Il-1β, Il-2, Il-5 and Il-10) were similar (Fig 4D). Crown-like structures in adipose tissue were seen in all cIMF and in 10/11 eIMF mice, but tended to occur more often in cIMF (Fig 4E; epididymal 8.0±8.8 versus 5.0±6.9; perirenal 9±10 versus 10±19). This difference was not statistically significant.

## Discussion

In the present work, we studied the long-term effects of early life exposure (PN16-42) to eIMF versus cIMF on body weight and body compositional development into adulthood when animals were continuously challenged to a HFD, as well as its effects on liver and adipose tissues size and function (Fig 1). Previously, eIMF with a different lipid composition was found to program mice for a lower body fat accumulation when they were challenged with a HFD up to PN126 (6). In the current study, IMFs had a different lipid composition and HFD exposure was extended to PN168. Similar programming effects were seen up to PN126, whereafter differences in body weight and composition disappeared upon continued HFD exposure (Fig 2). Interestingly, food intake, despite high variability, tended to be lower at PN154 in eIMF-fed mice (Fig. 2), strongly correlating with the slope of prior weight gain. This suggests that the differences in body weight can, at least in

part, be attributed to differences in food intake. The effects observed being transient may indicate that the programming response, i.e., the initial lower fat accumulation in adipose stores, can be overruled by a strong and persistent dietary challenge. In addition, our study suggests these programming effects, due to early life eIMF exposure, occurred regardless of the lipid composition of the fat globules, and is rather caused by the physicochemical structure of the lipids, i.e., globule size and phospholipid coating (10). In humans and mammals, the amount of lipids in (mature) milk, despite highly variable diets, is remarkably stable (26, 27). The size of milk fat globules, as well as the milk TG content, seem to be tightly regulated and species-specific (28), and greatly impact the absorption kinetics of breast milk lipids (29, 30). Testing eIMF (large and phospholipid coated fat globules) versus cIMF (small, uncoated fat globules) in adult men resulted in a different postprandial response upon a single bolus intake: an earlier postprandial glucose and insulin time-to-peak, an earlier non-esterified fatty acids (NEFA) time-to-nadir, and a later cholecystokinin time course (30). We hence speculate that fat globule size and phospholipid coating programs metabolic and tissue development induced by differential lipid uptake kinetics or post-absorptive lipid trafficking or tissue partitioning. Lipid uptake kinetics of eIMF, compared to cIMF, are likely to be more comparable to breastmilk.

Early-life feeding with eIMF compared to cIMF lowered liver weight in later life independent of body weight (Fig 3). The difference was not explained by mitotic index, or the degree of polyploidization. Hepatic triglyceride content had moderate variability without clear correlation with other parameters. Possibly the early-life diet primed hepatic tissue for a different response to a HFD challenge, resulting in a differential tissue growth. It is also possible, however, that the transient difference in body weight (or adipose tissue development) triggers a difference in liver size. Previously, a non-significantly higher liver weight had been seen in cIMF compared to eIMF and to an unchallenged control group (9). Additionally, a higher liver weight was seen upon early-life feeding with an IMF containing small compared to large lipid globules and challenging with a HFD (10). We hypothesize that postnatal liver development was changed due to a difference in post-absorptive lipid handling and trafficking. As mentioned earlier, eIMF is more rapidly absorbed compared to cIMF in adult men (30). In addition, a gavage of breastmilk, compared to standard formula, leads to a more rapid chylomicron (CM) production and a more rapid absorption of palmitic, arachidonic and docosahexaenoic acid in adult rats (29). Rapid absorption of protein-coated fat results in 3-fold larger CM diameter (31). However, we believe rapid lipid absorption only leads to larger CM when enteral phospholipid supply is limited, necessitating higher volume-to-surface area ratios (32). In contrast, CM diameter is lowered by biliary phospholipids (32). It is possible that breastmilk, and likewise eIMF, is rapidly absorbed and produces smaller CM than cIMF due to the dietary phospholipids provided with breastmilk and eIMF. The observation that an

IMF with large globules but lacking phospholipids does not program mice for less fat accrual in later life (10), adds weight to this notion. CM size and number affect the plasma half-life, as smaller particles have a larger relative surface area available to enzymes (33, 34), and are more quickly removed from the plasma via liver sieving (35). CM produced upon eIMF or HM ingestion are expected to have a different fractional clearance rate than CM upon cIMF ingestion. It is tempting to speculate that a more rapid absorption plus utilization, opposed to storage, of breastmilk and eIMF derived lipids in early life programs metabolism and fat accumulation capacity towards an advantageous trait for later-life health.

The effect on fat pad weights and adipocyte diameter was independent of body weight and composition (Fig 4). Previous studies showed that at PN98 the epididymal fat pad was smaller in eIMF-fed mice (9, 10), whereas this difference between test groups had disappeared at PN126 (6). We observed a higher epididymal fat mass in eIMF at PN168, indicating a differential fat distribution in eIMF vs. cIMF, as the perirenal and inguinal fat pad were similar in mass. Counterintuitively, the larger epididymal fat pads seen in eIMF did not result in lower levels of adiponectin and did result in higher levels of inflammatory markers (Tnf-α, Il-6), Mcp-1, or resistin (Fig 4) as typically seen with larger visceral adipose tissue (36). Interestingly, we found no effect on glucose metabolism and homeostasis (Fig 2I). Previously, eIMF-fed mice had lower plasma leptin, resistin, glucose and HOMA-IR at PN126 (6), likely related to the lower fat mass. A lower fat accrual rate, transiently seen in eIMF, is advantageous to metabolic health. However, given the observed minor effects on adipose tissue at PN168, we think it is unlikely that adipose tissue initiates the programming effect, and merely is a logical consequence and trait of the programmed phenotype.

## Concluding remark

The present study shows that feeding a postnatal diet containing large phospholipid-coated lipid globules has transient effects on body fat accrual during prolonged exposure to HFD. These effects are limited in strength and robustness and can be overruled by (too) strong environmental features, such as continued high-fat diet feeding. Our findings indicate the robustness and the limits of early-life programming due to eIMF exposure in the employed mouse model. The observed programming effects are hypothesized to be due to a difference in fat absorption, and/or post-absorptive handling and trafficking in the body.

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**Conflict of interest:** B.J.M.v.d.H. is employed by Danone Nutricia Research. H.J.V. is a consultant for Danone Nutricia Research outside the submitted work, for which his institution is compensated financially.

**Authorship (contribution statements):** B.J.M.vdH., H.J.V. and O.R. formulated the research question and designed the study. O.R. and A.J. carried out the study. A.d. B. and O.R. analyzed the data. H.J.V., F.K., B.J.M.vd.H. and O.R. wrote the paper.

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Table 1. Nutrient composition of the programming diets (PN16-42) and the high fat diet (PN42-168). *: all in g/kg

| | | Control IMF | Experimental IMF | HFD |
|---|---|---|---|---|
| **Carbohydrate *** | | 609 | 618 | 396 |
| Mono/di-saccharides | | 225 | 235 | 172.8 |
| | Glucose | 3.7 | 3.4 | - |
| | Lactose | 134 | 144 | - |
| | Sucrose | 85 | 85 | 172.8 |
| | Other sugars | 2.6 | 2.4 | - |
| Polysaccharides * | | 380 | 380 | 172.8 |
| | Maltodextrin | 101 | 101 | 100 |
| | Corn starch | 280 | 280 | 72.8 |
| | Other | 0.84 | 0.68 | - |
| Fiber * | | 49.0 | 48.2 | 50 |
| | Cellulose | 32.0 | 32.0 | 50 |
| | Fructo-oligosaccharides | 1.7 | 1.4 | - |
| | Galacto-oligosaccharides | 15.3 | 14.3 | - |
| **Lipids *** | | 77.2 | 70.6 | 203 |
| | Vegetable fat | 37.5 | 32.9 | 25 |
| | Milkfat | 38.6 | 36.7 | - |
| | Other animal fat | 1.1 | 0.98 | - |
| | Lard | - | - | 177.5 |
| | Phospholipids | 0.084 | 1.1 | - |
| | Cholesterol | 0.12 | 0.12 | 0.20 |
| **Protein *** | | 199 | 198 | 200 |
| | Whey | 17.6 | 16.5 | - |
| | Casein | 181 | 181 | 200 |
| **Particle size** | D[4,3] (µm) | 0.81 ± 0.2 | 6.8 ± 0.2 | - |
| | D[3,2] (µm) | 0.43 ± 0.004 | 0.86 ± 0.1 | - |
| | Surface area ($m^2$/g) | 15 ± 0.2 | 7.7 ± 1.0 | - |

Table 2. Fatty acid composition of the programming diets (PN16-42). *: all in FA weight%

| | | Control IMF | Experimental IMF |
|---|---|---|---|
| **Saturated*** | | **44** | **42** |
| | 14:0 | 8.9 | 7.1 |
| | 16:0 | 26 | 25 |
| | 18:0 | 7.8 | 8.9 |
| | 20:0 | 0.28 | 0.32 |
| | 22:0 | 0.27 | 0.39 |
| | 24:0 | 0.17 | 0.26 |
| | 26:0 | 0.032 | 0.039 |
| **Mono unsaturated*** | | **36** | **39** |
| | 16:1ω7 | 1.2 | 1.1 |
| | 18:1ω7 | 1.9 | 1.9 |
| | 18:1ω9 | 33 | 35 |
| | 20:1ω9 | 0.38 | 0.42 |
| | 22:1ω9 | 0.080 | 0.13 |
| | 24:1ω9 | 0.053 | 0.074 |
| **Polyunsaturated*** | | **20** | **19** |
| | **ω-3 species** | **3.4** | **3.4** |
| | 18:3ω3 | 2.8 | 2.8 |
| | 20:5ω3 | 0.12 | 0.12 |
| | 22:6ω3 | 0.38 | 0.38 |
| | 22:5ω3 | 0.090 | 0.099 |
| | **ω-6 species** | **16** | **16** |
| | 18:2ω6 | 16 | 15 |
| | 18:3ω6 | 0.050 | *trace* |
| | 20:4ω6 | 0.44 | 0.43 |
| | 20:3ω6 | 0.091 | 0.12 |
| | 20:2ω6 | 0.046 | *trace* |
| | **Σ ω-6 / Σ ω-3 ratio** | **4.8** | **4.7** |
| | 20:3ω9 | 0.38 | 0.42 |

Table 3. Hepatic histological scoring and fatty-acid ratios of mice programmed with cIMF (n=12) or eIMF (n=11) and subsequently challenged to a HFD. Values represent means ± SD.

| | | | Control IMF | | Experimental IMF | |
|---|---|---|---|---|---|---|
| | | | mean | SD | mean | SD |
| Steatosis | Steatosis grade | | 2.5 | 0.5 | 2.1 | 1.0 |
| | Steatosis | % | 70 | 17 | 57 | 32 |
| | Location steatosis | | 12/12 central | | 8/10 central; 2/10 azonal | |
| | Hepatocytes with micro- vs. macrovesicular steatosis | % | 78 | 9 | 78 | 8 |
| | Ballooning | | Few, 2/12 | | Few, 2/11 | |
| | Lobular inflammation | | 1.0 | 0.5 | 1.1 | 0.6 |
| | NAFLD score | | 3.7 | 0.8 | 3.3 | 1.5 |
| | Biliary/oval cell hyperplasia | | 1.4 | 0.8 | 1.5 | 0.5 |
| Mitosis | Mitotic index | % | 4.3 | 2.0 | 4.3 | 2.7 |
| | Binuclear cells per field | n | 4.5 | 2.1 | 4.7 | 3.3 |
| | Central binuclear cells | n | 4.7 | 3.5 | 4.1 | 3.6 |
| | Mid/portal binuclear cells | n | 4.4 | 1.7 | 5.2 | 3.7 |
| Fatty acyl chain ratios | Σ ω-6 / Σ ω-3 | | 6.3 | 0.6 | 6.2 | 0.72 |
| | 16:1ω7 / 16:0 | | 0.13 | 0.021 | 0.12 | 0.037 |
| | 18:1 / 18:0 | | 8.5 | 2.6 | 7.9 | 2.6 |
| | 18:1ω9 / 18:0 | | 7.8 | 2.4 | 7.3 | 2.4 |
| | 18:1 / 16:1 | | 13 | 1.9 | 14 | 2.8 |
| | 22:4ω6 / 18:2ω6 | | 0.029 | 0.01 | 0.025 | 0.006 |
| | 20:4ω6 / 20:3ω6 | | 4.8 | 1.1 | 5.3 | 1.5 |
| | 18:3ω6 / 18:2ω6 | | 0.021 | 0.002 | 0.020 | 0.003 |

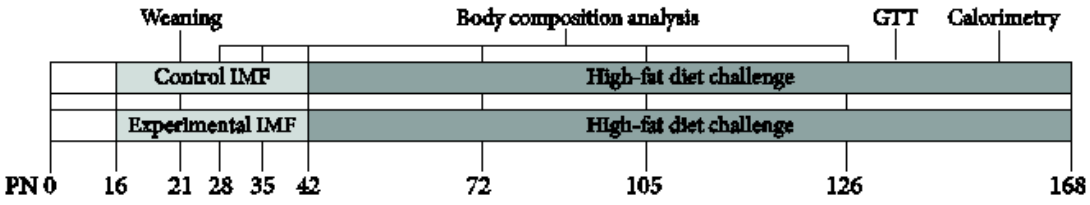


**Fig 1.** Study design from postnatal day (PN) 0 to 168 (n=12).

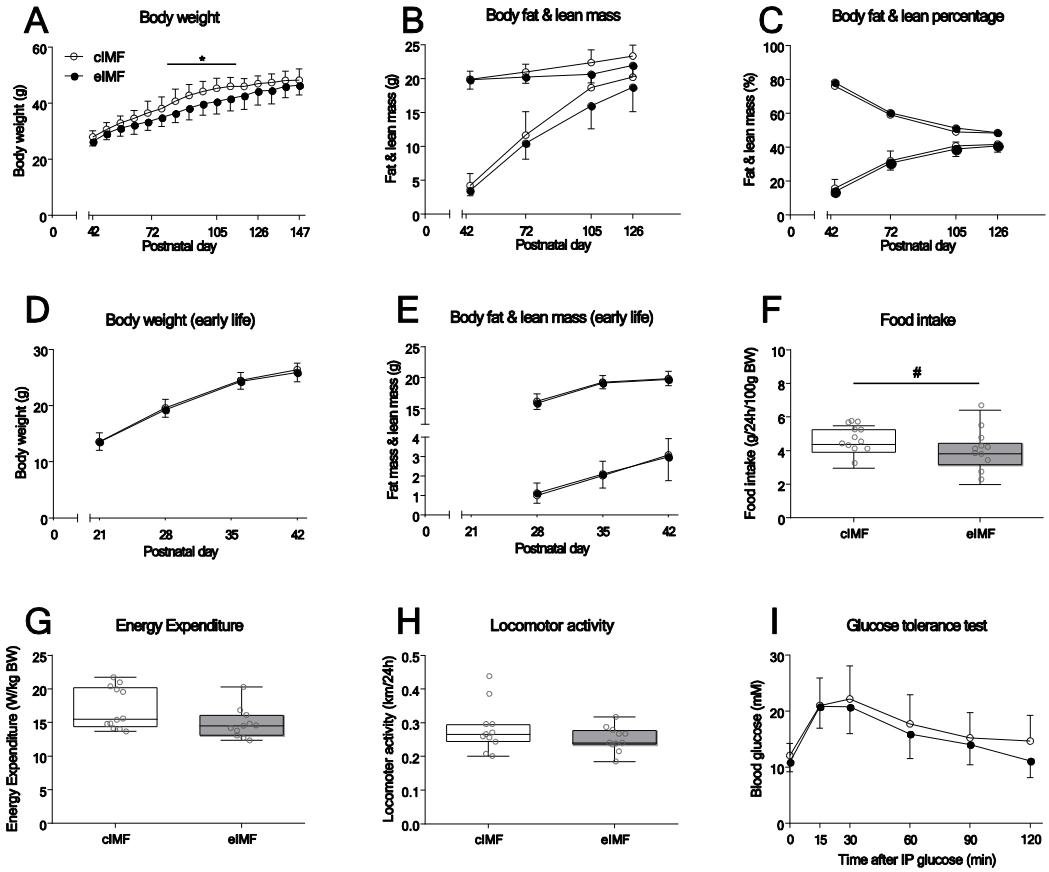


**Fig 2. Mice programmed with eIMF and challenged with a high-fat diet showed a transient lower body weight, lean mass and fat mass compared to animals programmed with cIMF .** Body weight (A), fat & lean mass (C) are expressed in absolute weights. The percentage of fat & lean mass (C) are expressed as % of body weight. Early-life body weight (D) and fat & lean mass (E) are expressed in absolute weights. Food intake (F), energy expenditure (G), and locomotor activity (H) was measured 3 times 24 h from PN154. Glucose tolerance at PN133 is shown as AUC (I). A-H: n=11-12; I: n=10-11; Mean±SD (A-E, I), Tukey boxplots and scatter plots (F-H); * $p<0.05$.

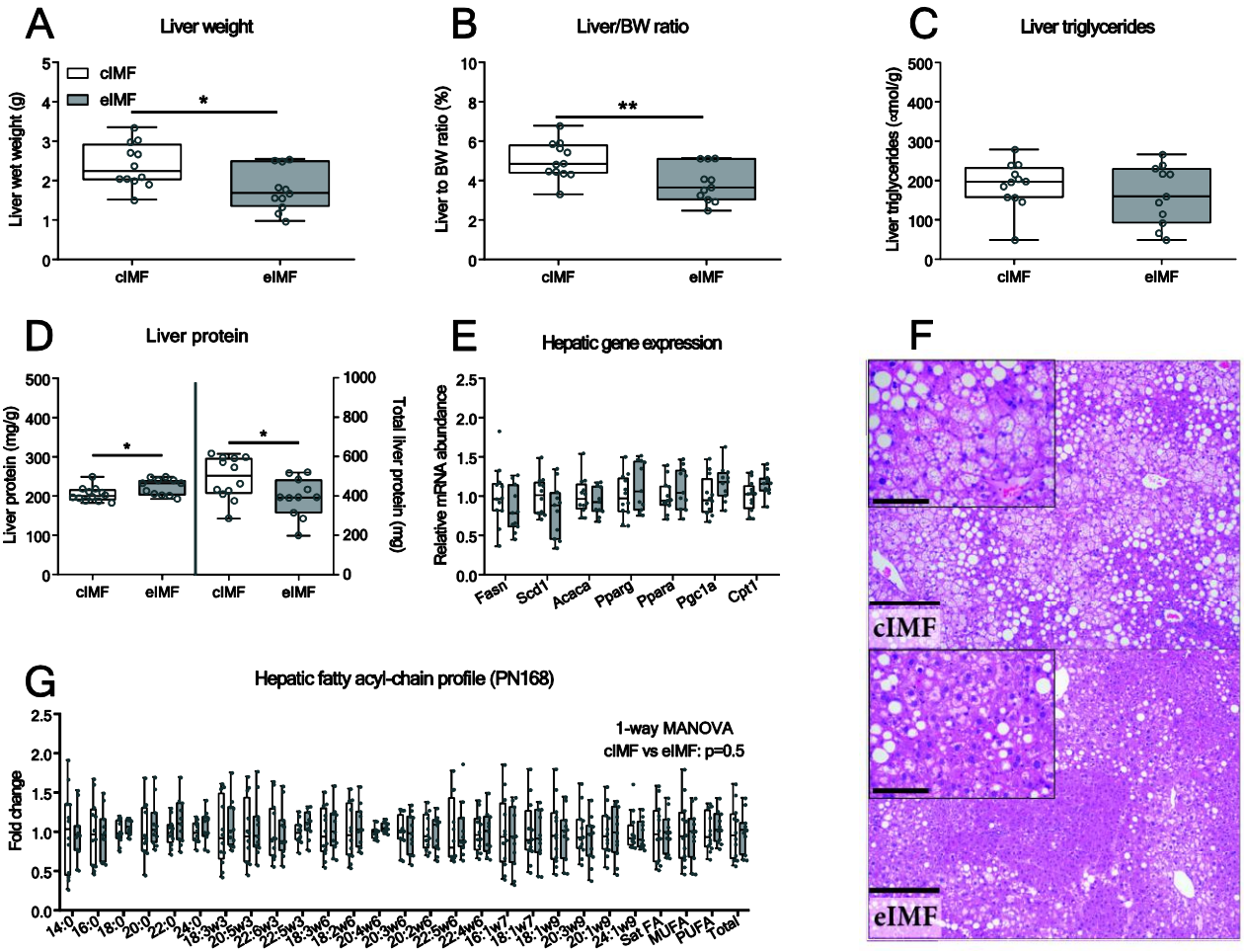


**Fig 3. Mice programmed with eIMF compared to cIMF showed a lower liver weight with a concurrent higher protein content without a shift in fatty acyl chain profile.** Dissection was performed at PN168. Liver weight (A) is expressed as wet weight. The liver to bodyweight ratio (B) is expressed as % of bodyweight. TG levels (C) are expressed per gram liver. Liver protein content (D) is expressed as mg per gram wet liver tissue. Hepatic mRNA levels (E) were normalized to cyclophilin. Fatty acyl chain profile is expressed as fold change compared to cIMF (G). Liver histology (H&E; F) showed a zonal distribution characterized by central microvesicular steatosis with mild to moderate mid-zonal macrovesicular steatosis. Bar: 250µm, inset bar: 100µm. n=11-12; Tukey boxplots and scatter plots; ** $p<0.01$; * $p<0.05$.

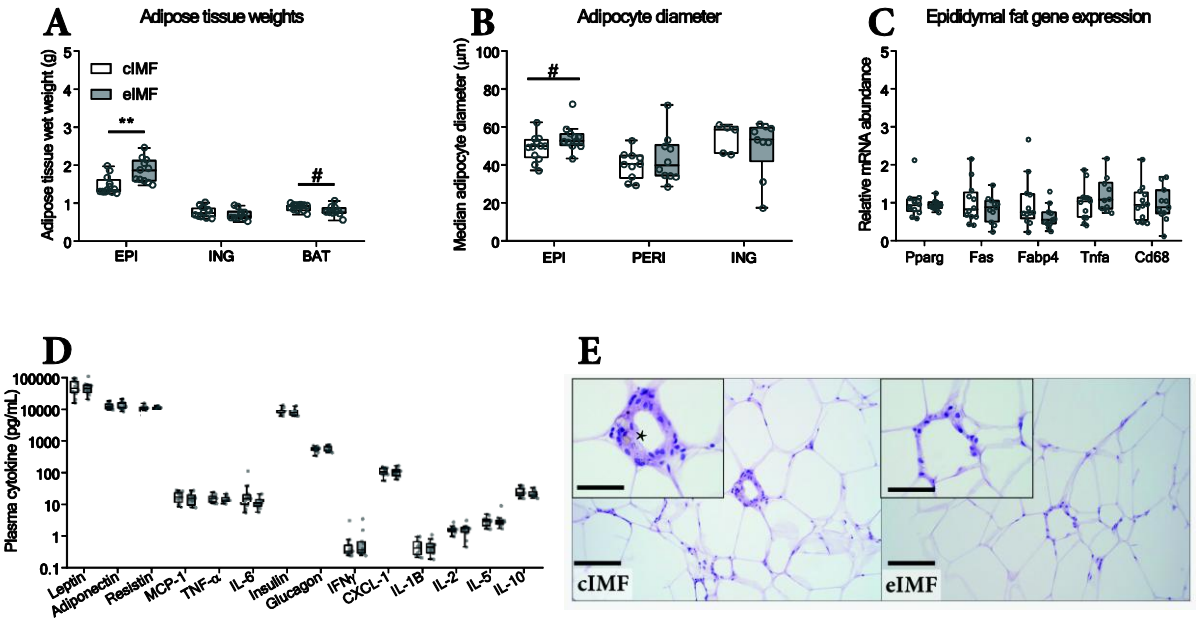


**Fig 4. Mice programmed with eIMF compared to cIMF showed an higher visceral adipose tissue weight and adipocyte cell diameter**. Epididymal (EPI) visceral, inguinal (ING) subcutaneous and interscapular (BAT) brown adipose tissue (A) are expressed as absolute weights. EPI, perirenal (PERI) and ING (B) adipocyte diameter was calculated (Adiposoft) and expressed as equivalent diameter. The epididymal fat gene expression (C) was normalized to 36b4 and shown as fold change. Plasma adipokines, glucostatic hormones and cytokines are expressed as pg/ml (D). Adipose (epididymal depot) pathology (E) characterized by crown-like structures composed of macrophages and other mixed inflammatory cells with lipofuscin (*) surrounding a necrotic adipocyte, bar: 100 µm, inset bar: 50 µm. Tukey boxplots and scatter plots. n=10-12 ** $p<0.01$; * $p<0.05$; # $p<0.10$.